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SO JOURNAL OF ANIMAL SCIENCE, (1993 Mar) 71 (3) 687-93.

L4 ANSWER 49 OF 66 MEDLINE DUPLICATE 17
AU Kolb A F; Albang R; Brem G; Erfle V; Gunzburg W H; Salmons B
TI Characterization of a protein that binds a negative regulatory element in the mammary-specific whey acidic protein promoter.
SO BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, (1995 Dec 26) 217 (3) 1045-52.

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AU Petitclerc D; Attal J; Theron M C; Bearzotti M; Bolifraud P; Kann G; Stinnakre M G; Pointu H; Puissant C; Houdebine L M
TI The effect of various introns and transcription terminators on the efficiency of expression vectors in various cultured cell lines and in the mammary gland of transgenic mice.
SO JOURNAL OF BIOTECHNOLOGY, (1995 Jun 21) 40 (3) 169-78.
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Development of a Recombinant Bovine Leukemia Virus Vector for Delivery of a Synthetic Bovine Growth Hormone-Releasing Factor Gene into Bovine Cells[1]

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ABSTRACT: Continuous intravenous infusion of bovine growth hormone-releasing factor (bGRF) increases milk synthesis in dairy cattle by as much as 46%. We have begun to develop a system for delivery and expression of a synthetic bGRF gene in cultured bovine cells using the provirus of the bovine leukemia virus (BLV). The gene encoding synthetic bGRF, constructed from eight overlapping oligonucleotides, was fused to the whey acidic protein promoter (WAP) or the mouse mammary tumor virus promoter (MMTV). These plasmids, termed pWAP.GRF and pMMTV.GRF, were able to induce transcription of bGRF upon transfection into Madin-Darby bovine kidney (MDBK) cells and induction with a lactogenic hormonal milieu (prolactin, hydrocortisone, triiodothyronine, insulin) or dexamethasone. When these constructs were cloned into a BLV vector in place of its oncogenic region, and transfected into MDBK cells, bGRF was expressed. Virus particles were prepared from these cultures and used to deliver the bGRF gene by viral infection into fresh MDBK cells. Northern blot analysis of MDBK total RNA revealed a fivefold higher level of expression of bGRF mRNA in transfected cultures than in virally infected cells, and no expression was detected in control cultures. The bGRF peptide was detected in both cell extracts and media samples from transfected cultures but was not detected in cell extracts or media samples from virally infected cells. This provirus construct may prove useful as a delivery system for peptides into cattle.

Key Words: Growth Hormone Releasing Factor, Bovine Leukemia Virus, Retroviridae

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Introduction

Continuous delivery of exogenous peptides into food-producing animals to alter their carcass composition or milk production is difficult. Peptides are usually injected either intravenously, subcutaneously, or intramuscularly to prevent the hydrolysis that would normally occur by the oral route of delivery. Response of the animal to a peptide may be inefficient because of the short half-life of the peptide after a single injection. If the gene for a particular peptide could be stably inserted into an animal under the appropriate regulatory control, then it would be possible to deliver the peptide on demand and to bypass the requirement for an invasive delivery system.

We have selected the bovine growth hormone-releasing factor (bGRF) gene as a model system to study delivery of peptides because this peptide is galactopoietic (Dahl et al., 1990). It has been necessary to infuse bGRF peptide intravenously for a maximal response in growth hormone secretion to occur (Moseley et al., 1985). Up to a 46% increase in milk yield has been demonstrated upon infusion of bGRF into dairy cows (Dahl et al., 1990); therefore, this peptide is useful for these experiments because it has a readily detected response and a large metabolic effect. We have begun to investigate alternate methods of delivery for bGRF by using the bovine leukemia virus (BLV) retroviral vector. Retroviral vectors have been used successfully for delivery and expression of foreign genes into both mice (Jaenisch and Croker, 1975; Jähner and Jaenisch, 1980; Rubenstein et al., 1986) and larger domestic animals (Squire et al., 1989). Therefore, the objective of our research was to use a retroviral vector as a method of

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delivery of a synthetic bGRF gene into bovine cells in tissue culture and to examine expression of this gene under the control of two hormonally responsive promoters.

Experimental Procedures

Tissue Culture Cells. Madin-Darby bovine kidney (MDBK) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were propagated in Eagle minimal essential medium (EMEM) with Earle salts (GIBCO Laboratories, Santa Clara, CA) and 10% horse serum with 1× penicillin/streptomycin solution (5,000 units penicillin/mL, 5 mg of streptomycin/mL in .9% NaCl; Sigma Chemical, St. Louis, MO). Cultures were maintained in 100-mm cell culture dishes at 37°C with an atmosphere of 5% CO_2.

Bovine Growth Hormone-Releasing Factor Gene Construction. The nucleotide sequence used for construction of the synthetic bGRF gene was derived from the published amino acid sequence of bGRF (Esch et al., 1983). Eight overlapping oligonucleotides (oligos) were synthesized for this construct (Figure 1). The sequence of each oligonucleotide was determined by Maxam-Gilbert sequence analysis (Maniatis et al., 1982). Each oligonucleotide was annealed to its complementary strand partner and purified on a 1.6% agarose gel to obtain four cassettes. The first two cassettes (containing oligos I and III and II and IV) were ligated using 1 unit T_4-DNA ligase at 16°C overnight in ligation buffer (50 m*M* Tris [pH 7.4], 10 m*M* $MgCl_2$, 10 m*M* dithiothreitol [DTT], .1 mg/mL of BSA, fraction V) and the resulting product purified by agarose gel electrophoresis. The next two cassettes (containing oligos V and VI and VII and VIII) were ligated and purified as above. Finally, the two combined cassettes were ligated, purified on a 1.6% agarose gel, and then ligated into the *Eco*RI site of the plasmid pUC19. The resulting plasmid was called pbGRF. Because of the design of the bGRF fragment, ligation into the vector eliminated the *Eco*RI site at the 5′ end of the gene but maintained the 3′ *Eco*RI site for use in further subcloning. The correct sequence of this construct was verified by DNA sequence analysis using Sequenase (United States Biochemical, Cleveland, OH).

Selection of Promoters and Subcloning Strategy. The whey acidic protein (WAP) promoter (Campbell et al., 1984) and the mouse mammary tumor virus (MMTV) promoter (Majors and Varmus, 1983) were obtained from the ATCC (ATCC #63005 and 45007). Both promoters increase transcription in response to the addition of prolactin in tissue culture (Campbell et al., 1984; Munoz and Bolander, 1989). In addition, the MMTV promoter increases transcription in response to the addition of dexamethasone (Buetti and Diggelmann, 1981). The .5-kb (kilobase pair) WAP promoter was excised from its original vector with *Xho*I and *Kpn*I restriction enzymes (Boehringer Mannheim, Indianapolis, IN) and ligated into the *Kpn*I site of pbGRF overnight at 16°C with 1 unit of T_4-DNA ligase and 2 m*M* ATP in the same ligation buffer described above. The resulting DNA fragment was made circular by filling in the remaining restriction half-sites (*Xho*I, *Kpn*I) with 2 units of Klenow enzyme and 2 m*M* of each of the four deoxynucleotides for 15 min at 37°C, precipitated in ethanol, resuspended in ligation buffer, and ligated at 22°C overnight with 1 unit of T_4-DNA ligase and 2 m*M* ATP to generate the construct pWAP.GRF (Figure 2). The 1.3-kb MMTV promoter was excised from its original vector with *Bam*HI and subcloned into the *Bam*HI site of pbGRF. Both plasmid constructs were transformed into *E. coli* strain DH5-alpha. The correct orientation of each promoter relative to bGRF was determined by DNA sequence analysis using Sequenase (Figure 2).

Bovine Leukemia Virus Subclones. Promoter-bGRF constructs were subcloned into the bovine leukemia

Synthetic Bovine GRF Gene

```
                         I                                                  III
AATTGATGTA CGCTGACGCT ATCTTCACTA  ACTCTTACCG TAAAGTTCTG GGTCAACTGT CTGCTCGTAA ACTGCTGCAA
      CTACAT GCGACTGCGA TAGAAGTGAT  TGAGAATGGC ATTTCAAGAC CCAGTTGACA GACGAGCATT TGACGACGTT
                      II                                  IV

                    V                                          VII
GACATCATGA ACCGTCAGCA GGGTGAACGT AACCAGGAAC AGGGTGCTAA AGTTCGTCTG TCTTAGTAAC GCGCGTTCTC AATAAAG
CTGTAGTACT TGGCAGTCGT CCCACTTGCA TTGGTCCTTG TCCCACGATT TCAAGCAGAC AGAATCATTG CGCGCAAGAG TTATTTCTTAA
           VI                                                   VIII
```

Figure 1. Sequence of synthetic bovine growth hormone-releasing factor (GRF) gene. The eight oligonucleotides used for construction are noted above and below the sequence. Arrows signify the continuation of the construct. Nucleotides were determined by extrapolation from a known amino acid sequence (Esch et al., 1983).

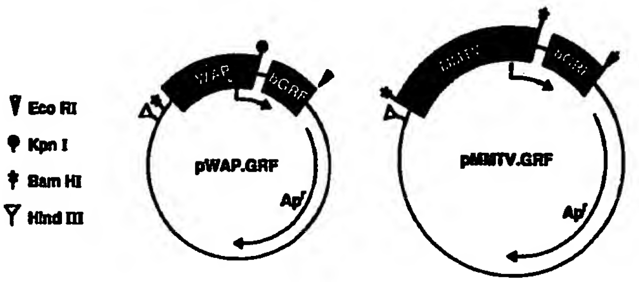

Figure 2. Intermediate plasmid constructs using the synthetic bovine growth hormone releasing factor (bGRF) gene and lactation-signal-responsive promoters. Restriction sites used for cloning and their plasmid map positions are as shown. WAP = Whey acidic protein promoter, MMTV = mouse mammary tumor virus promoter.

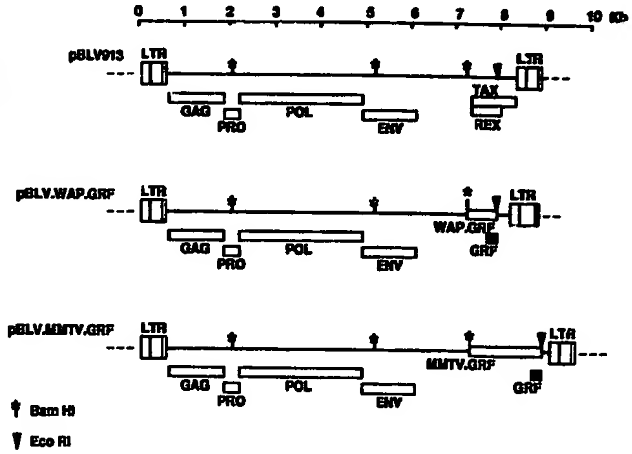

Figure 3. Genetic maps of the bovine leukemia virus (BLV) provirus clones. The pBLV-913 is the original provirus clone. The pBLV.WAP.GRF contains the whey acidic protein promoter fused to the bovine growth hormone releasing factor gene (WAP.GRF) fragment excised from pWAP.GRF and subcloned into the area shown in BLV-913. The pBLV.MMTV.GRF contains the mouse mammary tumor virus promoter fused to the bovine growth hormone releasing factor gene (MMTV.GRF) fragment excised from pMMTV.GRF and subcloned into the similar map area of BLV-913. The subclones are, therefore, missing the TAX/REX portion of the original BLV genome. LTR = long terminal repeats. The abbreviations GAG, PRO, POL, and ENV denote gene products coding for both regulatory and structural retrovirus proteins. Gene products are denoted by the open boxes and bGRF peptide by the solid box.

virus (BLV) vector pBLV-913 (Figure 3) (Derse and Martarano, 1990). The WAP.GRF fragment was excised from pWAP.GRF with *Bam*HI and *Eco*RI. The MMTV.GRF fragment was excised from pMMTV.GRF with an *Eco*RI and a partial *Bam*HI digest because of the presence of additional *Bam*HI sites (Majors and Varmus, 1983). Both fragments were purified by agarose gel electrophoresis (Maniatis et al., 1982). The pBLV-913 was fully digested at the unique *Eco*RI site at position 7925 in the 3′ end of the viral genome (Figure 3) then partially digested with *Bam*HI (Sagata et al., 1985). Viral DNA digested at the *Bam*HI site at 7203 in the viral genome was 10,678 base pairs (bp) in length and was isolated by agarose gel lectrophoresis. Fragments were cut from the gel with a razor blade, squeezed through a 1-mL syringe, and extracted with phenol to obtain purified DNA. This viral DNA was missing the *tax/rex* portion of the viral genome, and therefore, could not replicate and was non-oncogenic (Derse and Martarano, 1990). The promoter-GRF fragments were recombined with the *tax/rex* deletion BLV subclones by ligating overnight as described in the previous section and transformed into *E. coli* strain DH5-alpha. Resulting clones (pBLV.WAP.GRF and pBLV.MMTV.GRF) were characterized in detail by restriction enzyme mapping (Figure 3).

Transfection of pMMTV.GRF and pWAP.GRF Subclones. Transfection of MDBK cells was performed by th calcium-phosphate precipitation method (Stuart et al., 1985). Ten micrograms of each plasmid or no plasmid was transfected into 80% confluent MDBK cells. Calcium-phosphate precipitation proceeded for 12 h, and the medium was removed and fresh medium added. At 36 h after transfection, 1 μg/mL of dexamethasone was added to one f the two plates containing pMMTV.GRF, and a lactogenic hormone milieu (15 μg/mL of prolactin, 1 μg/mL of hydrocortisone, 40 ng/mL f 3,3′,5′-triiodo-L-thyronine, and 400 ng/mL of insulin) was added to one of two plates containing pWAP.GRF. At 48 h after transfection, total RNA was prepared. Control transfections were prepared in identical fashion except no DNA was added to cells.

Transfection/Infection with Bovine Leukemia Virus Subclones. The BLV-GRF constructs were cotransfected into MDBK cells by the calcium-phosphate procedure using a helper plasmid, pBLPX-RSPA (Derse and Martarano, 1990), which contains the *tax/rex* portion of the BLV genome under the control of the Rous sarcoma virus promoter. One hundred micrograms of each BLV-GRF plasmid was transfected into 80% confluent MDBK cells, along with 50 μg of the helper plasmid. Transfecti n proceeded f r 12 h, then the medium was replaced with fresh medium. At 48 h after transfection, cells were stimulated with either dexamethasone or lactogenic hormonal milieu as described above. All media used for stimulation were lacking horse serum (which would rapidly degrade bGRF peptide) but did contain the protease inhibitor alpha$_2$-macroglobulin to minimize th effects f pro-

teolytic enzymes that remained from residual serum. At 60 h after transfection, the growth medium was collected from each plate and viral particles were purified by sterile filtration through a .22-μm syringe filter (Gelman Sciences, Ann Arbor, MI). Two milliliters of the filtrate was frozen for protein analyses (described later) and 8 mL was used to infect fresh MDBK cells. Total RNA was prepared from the transfected MDBK cells as described below. The MDBK cells were incubated in the presence of growth medium filtrate to allow infection (16 h), then new medium was added. At 72 h after infection, media were changed to include either dexamethasone or lactogenic hormonal milieu as described above. At 84 h after infection, growth media were frozen for protein analysis. Total RNA was also prepared from the infected MDBK cells as described below.

Preparation and Analysis of RNA. Preparation of RNA was by the proteinase K method as previously described (Stuart et al., 1985). The RNA (15 μg per lane as quantified spectrophotometrically) was subjected to electrophoresis for 4 h at 50 V on 1.5% agarose, 2.2 *M* formaldehyde gels in 1× MOPS buffer (Stuart et al., 1985). Gels were stained by placing them in .1 *M* ammonium acetate with 5 μg/mL of ethidium bromide for 30 min. The RNA sizes were determined by comparison to a .24- to 9.5-kb RNA ladder (Bethesda Research Labs, Bethesda, MD). Transfer to Hybond N solid support (Amersham, Arlington Heights, IL) was accomplished by soaking the gel for 45 min in 50 m*M* NaOH, 10 m*M* NaCl, neutralizing the gel by soaking in .1 *M* Tris-HCl (pH 7.5) for 45 min, and then Northern blotting as described (Maniatis et al., 1982). Transfer was m nitored by viewing the Hybond N under ultraviolet light and by restaining the agarose gel. In all cases complete transfer was achieved. The RNA was cross-linked to the solid support by exposure to ultraviolet light for 2 min on a transilluminator (Spectroline Model TR-302, 302 nm ultraviolet).

The *Bam*HI/*Eco*RI bGRF fragment isolated from pbGRF was labeled to high specific activity using a commercial random-primed labeling kit (Boehringer Mannheim), and ^{32}P-CTP (3,000 Ci/mmol) (New England Nuclear, Wilmington, DE). This probe was added to a nonaqueous solution and hybridized at 42°C for 20 h (Stuart et al., 1985). After hybridization, filters were washed for 1 h in 2× SSC (.15 *M* NaCl, .015 *M* sodium citrate), .1% SDS (sodium dodecyl sulfate) at 65°C, followed by 30 min in .3× SSC, .1% SDS at 65°C. Filters were exposed to x-ray film (X-OMAT, AR, Eastman Kodak, Rochester, NY) at −70°C with intensifying screens. X-ray films were analyzed with a densitometer (Mod l 710 Fluorometer/Densitometer, Ciba-Corning, Oberlin, OH).

Bovine Growth Hormone-Releasing Factor Immunoblot Analysis. Immunoblots of bGRF peptide were prepared by electrophoresis of concentrated cell extracts and growth media prepared from transfected/infected MDBK cells using SDS-PAGE by the Hoefer procedure for low-molecular-weight protein gels (Giulian and Graham, 1990) with the foll wing modifications: 50 m*M* DTT was added to the sample buffer, and samples were boiled for 5 min before they were loaded onto the gels. Two gels were used to resolve identical sets of protein samples. One gel was stained overnight in Coomassie brilliant blue G in 50% methanol, 10% acetic acid, then destained in 10% methanol, 10% acetic acid for viewing of samples and molecular weight markers for peptides (2,500 to 17,000) (Sigma). Proteins from the second gel were transferred via capillary action to nitrocellulose. The gel was equilibrated in 50 m*M* Tris-HCl (pH 9.5) for 10 min and placed on two sheets of Whatman #3 chromatography paper that functioned as wicks for the reservoir buffer (50 m*M* Tris-HCl 9.5). Zetabind membrane (charged nylon) (CUNO, Meriden, CT) was cut to size, hydrated, and then placed on the gel. Two sheets of Whatman chromatography paper were placed on the membrane and a stack of paper towels placed on the top of them. Passive transfer proceeded for 39 h with a change of transfer buffer and paper towels at 18 h.

The primary antibody in the immunoblot analysis was a polyclonal rabbit anti-serum prepared against purified bGRF (1–44)-NH_2 (M. B. Kamdar, Upjohn, Kalamazoo, MI). The antibody was characterized by M. B. Kamdar. Cross-reactivity studies were performed by RIA analysis using rabbit anti-bGRF (1–44)-NH_2 at an initial titer of 1:40,000 and ^{125}I-hGRF (1–44)-NH_2 (Amersham, Lot #53,55) as tracer. The antibody cross-reacted completely with bGRF (1–44)-NH_2 and GRF variants with conserved 1–29 amino acid regions, but cross-reacted only minimally (< .01 to 8%) with GRF variants containing < 29 amino acids. The secondary antibody was goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Sigma # A8025). Nonspecific binding of antibody to the solid support was reduced by blocking with 10% BSA, .5% nonfat dry milk, .05% NaN_3 in PBS for 6 h at 45°C. The blocking solution was decanted, and 5μg/mL of the primary antibody was added in (TBS)-Tween 20 (50 m*M* Tris [pH 7.5], .05% Tween 20, .87% NaCl) with 5% fetal calf serum for 2 h at room temperature. The blot was then washed five times with TBS-Tween 20 for 15 min each. The secondary antibody was then added to the TBS-Tween 20 at a 1:2,500 dilution and incubated at room temperature for 2 h. Th bl t was washed five times in TBS-Tween 20 for 15 min each. Th blot was then rinsed for 15 min in AP buffer (.1 *M* Tris 9.5, .1 *M* NaCl, 5 m*M* $MgCl_2$) and developed in NBT/BCIP reaction mix (33 mg of nitrobluetetrazolium, 16.7 mg of bromochloroindoyl phosphate in 100 mL of AP buffer). The color reacti n was stopped by removal of developer and addition f distilled water.

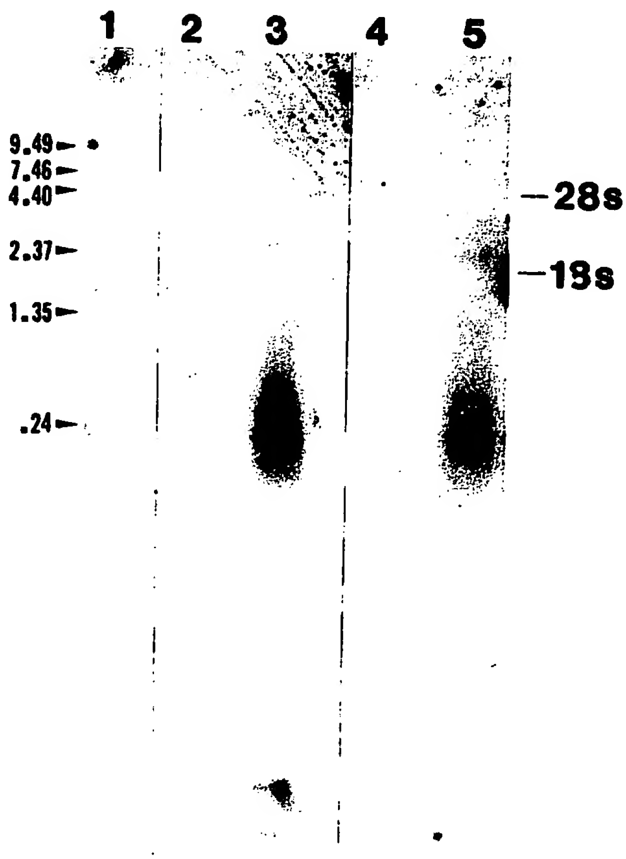

Figure 4. Northern blot analysis of steady-state levels of RNA produced from transient transfections of pWAP.GRF and pMMTV.GRF. The positions of low-molecular-weight RNA markers are noted on the left of the figure. Positions of the 28S and 18S ribosomal RNA are noted to the right of the figure. Lanes: 1) mock-transfected MDBK, 2) pWAP.GRF transfected MDBK, n nstimulated, 3) pWAP.GRF-transfected MDBK, stimulated with lactogenic hormonal milieu, 4) pMMTV.GRF-transfected MDBK, nonstimulated, and 5) pMMTV.GRF-transfected MDBK, stimulated with dexamethasone.

Results

Expression of Bovine Growth Hormone-Releasing Factor in Madin-Darby Bovine Kidney Cells Transfected with pWAP.GRF and pMMTV.GRF. To analyze the inducibility of the MMTV and WAP promoters in a bovine cell line, we transfected the plasmids pWAP.GRF and pMMTV.GRF into MDBK cells and induced transcription by the addition of dexamethasone or lactogenic hormonal milieu to the tissu culture media. The RNA prepared from these cells was subjected to Northern blot analysis (Figur 4) using a radioactively labeled purified bGRF fragm nt as probe. The presence of bGRF mRNA in the induced cultures showed a clear transcriptional activation of both promoter-bGRF gene constructs.

Expression of Bovine Leukemia Virus Subclones. Cotransfection of BLV subclones, pBLV.WAP.GRF and pBLV.MMTV.GRF, into MDBK cells with a helper plasmid that contains the *tax/rex* genes under control f the Rous sarcoma virus promoter allowed the expression of bGRF from the transfected cultures as well as preparing virus for infection of other cells. Detectable bGRF expression occurred only upon transfection of large amounts of the BLV subclones (100 μg of plasmid per 2 × 10^6 cells) along with a large amount of the helper plasmid (50 μg of plasmid per 2 × 10^6 cells). The bGRF was clearly detectable in the transfected cultures, whereas in the cultures infected with virus prepared from the transfected plates (and minus the helper plasmid), detectable levels of expression were 80% lower (Figure 5) for BLV.WAP.GRF and just above background levels for BLV.MMTV.GRF.

Expression of bGRF peptide was monitored using immunoblot analysis (Figure 6). The bGRF peptide was produced from the transfected cultures at a detectable level and was found in both cell extracts and in one tissue culture medium, whereas virally infected cultures did not produce enough protein for detection.

Discussion

We are interested in development of alternative methods for delivery of peptides into animal systems. It is clear that for useful peptide delivery to occur in an animal system, expression of the gene for th peptide must be tied to a regulatory control found in the animal. Otherwise constant expression of peptides could have deleterious effects, as has been noted for other delivery systems (Squire et al., 1989). Our current studies show that the BLV vector can be used to express a small peptide hormone gene under the control of exogenous promoters in a bovine tissue culture cell system. The use of the WAP and MMTV promoters has allowed us to transfect the bGRF gene into MDBK cells and to achieve transient inducibl expression by lactogenic hormones. Also, we were abl to express these constructs after subcloning them into the BLV provirus and cotransfecting them with the *tax/rex* helper plasmid. Virus infection produced a much lower level of RNA expression, and no detectabl protein expression. Each experiment was reproduced at least once, and the data were essentially similar between studies. After these assays were performed we checked high-m lecular-weight DNA prepared from these cultures by a sl t-blot analysis and determined that delivery of viral sequences had occurred (data not shown). It is possible that even though viral DNA was delivered to the cells, gen dosage may not have been equivalent between transfected and infected cultures. Ineffici nt expression

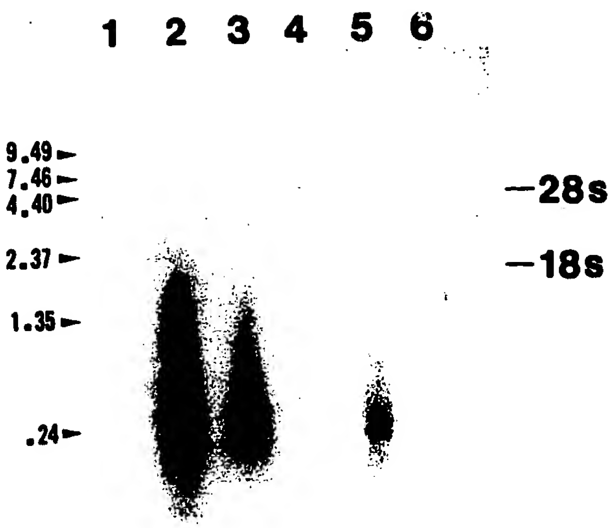

Figure 5. Northern blot analysis of steady-state levels of RNA produced from cotransfections of the bovine leukemia virus (BLV) subclones and from cells infected with virus preparations. Lanes: 1) mock-transfected Madin-Darby bovine kidney cells (MDBK), 2) pBLV.WAP.GRF cotransfected with pBLPX-RSPA and stimulated with lactogenic hormonal milieu, 3)pBLV.MMTV.GRF cotransfected with pBLPX-RSPA and stimulated with dexamethasone, 4) mock-infected MDBK, 5) MDBK cells infected with virus prepared fr m pBLV.WAP.GRF-transfected cells and stimulated with lactogenic hormonal milieu, and 6) MDBK cells infected with virus prepared from pBLV.MMTV.GRF-transfected cells and stimulated with dexamethasone.

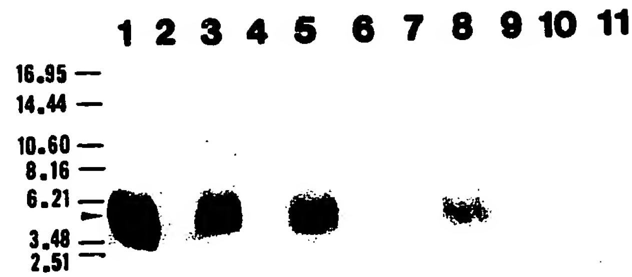

Figure 6. Immunoblot analysis of bovine growth hormone-releasing factor (bGRF) peptide production from transfected/infected Madin-Darby bovine kidney (MDBK) cells induced with lactogenic hormonal milieu or dexamethasone. Protein samples were prepared from either cell extracts or media taken from transfected or infected cells. Samples were concentrated by lyophilization, resolved on denaturing low-molecular-weight polyacrylamide gels, and blotted to nitrocellulose as described in Materials and Methods. Positions of low-molecular-weight protein markers are denoted to the left of the figure. Lanes: 1) pure GRF peptide, 2) mock-transfected cell extract, 3) pBLV.WAP.GRF-transfected cell extract, 4) pBLV.WAP.GRF virus infection cell extract, 5) pBLV.MMTV.GRF-transfected cell extract, 6) pBLV.MMTV.GRF virus-infected cell extract, 7) mock-transfected cell media, 8) pBLV.WAP.GRF-transfected cell media, 9) pBLV.WAP.GRF virus-infected cell media, 10) pBLV.MMTV.GRF-transfected cell media, and 11) pBLV.MMTV.GRF virus-infected cell media.

may also have happened because of deletions or point mutations that may have occurred during virus production. Furthermore, it is unknown what effect the missing *tax*/*rex* gene products may have on transcription of a foreign promoter in the BLV genome, or translation of a foreign gene product. It is also possible that virus infection is inefficient in this cell system, although it is known that the MDBK cell is a high-titer BLV producer after it is transfected with the riginal provirus (Ders and Martarano, 1990). Future studies should involve testing a number of bovine cell types f r efficient delivery of genes and expression, as w ll as the development of high-titer virus-producing cell lines. Although we believe this system is worth pursuing for delivery of peptides, one of the obvious drawbacks of the BLV system at this time is that it is a complex retrovirus that still requires further study of its genetic components and their function.

The usefulness of viral vector delivery systems for delivery of exogenous genes into animal systems is currently being evaluated by a number of investigators. For example, the Moloney murine leukemia virus has been extensively characterized and used successfully for many years to produce transgenic mice (Jaenisch and Croker, 1975; Jähner and Jaenisch, 1980; Rubenstein et al., 1986). However, difficulty in gene delivery and expression of this system into bovine cells in tissue culture (Squir et al., 1989) convinced us to attempt to develop a bovin viral vector for this purpose. Previous work has shown the bovine leukemia virus provirus to be amenable to the construction and use of its genom as a viral vector in cell culture (Derse, 1988; Derse and Martarano,

1990). This virus contains two regulatory proteins, *tax* and *rex*, in the 3′ portion of its genome (Ders , 1988). The *tax* protein is essential for viral replication by acting together with *cis*-acting sequences in the long terminal repeat sequences to regulate initiation of transcription (Derse, 1988; Derse and Martarano, 1990). Unfortunately, *tax* is known to induce chromosomal damage to the host cell system (Tanaka et al., 1990) and, therefore, cannot be used to construct cell lines that stably express the *tax/rex* proteins. Development of a high-titer system may, therefore, have to make use of a BLV provirus that contains a selectable marker. The *rex* protein acts to modulate the amount of structural protein mRNA (Derse, 1988; D rse and Martarano, 1990). Removal of the *tax/rex* portion of the genome produces a reliable nononcogenic suicide vector. This could prove useful in future studies for delivery of foreign genes into animal cell culture (Derse and Martarano, 1990).

Implications

Delivery and inducible expression of a small synthetic peptide was obtained upon transfection of the pBLV.MMTV.GRF and pBLV.WAP.GRF plasmids. Virus containing the synthetic bovine growth hormone-rel asing factor gene was produced, and this was used to infect bovine cells. The provirus of bovine leukemia virus seems to be an appropriate choice for the future dev lopment of a gene delivery system for peptides into bovine cells.

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